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The first evidence for the bacterial methylation of tin from inorganic Sn(IV) was presented in 1973 and recently has been verified by other investigators. Recent studies with Pseudomonas strain 244, first employed in the initial NBS observations, have indicated that the volatile methylated species produced by this organism from Sn(IV), and to a considerably lesser extent from Sn(II),

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BIOTRANSFORMATION OF TIN

W. R. Blair[1,2], J. A. Jackson[1], G. J. Olson[1,3], F. E. Brinckman[1], and W. P. Iverson[1]

ABSTRACT

The first evidence for the bacterial methylation of tin from inorganic Sn(IV) was presented in 1973 and recently has been verified by other investigators. Recent studies with Pseudomonas strain 244, first employed in the initial observations, have indicated that the volatile methylated species produced by this organism from Sn(IV) and to a considerably lesser extent from Sn(II), include tetramethyltin (Me_4Sn) and a number of hydridic methylstannanes (Me_nSnH_{4-n}, n = 2,3). Subsequently, using hydridization coupled with a new purge/trap gas chromatographic method, methylated tin compounds (methylstannanes) have been found in the Chesapeake Bay, as both volatile and non-volatile species [$(CH_3)_nSnH_{4-n}$ and $(CH_3)_nSn^{(4-n)+}$, respectively]. Concentrations have ranged from 0 to 930 ng L^{-1}. Studies on the biological transformation of organotin compounds have so far indicated that there are no significant biotransformations of tributyltin moiety, Bu_3Sn^+ (TBT). TBT-resistant organisms from the Chesapeake Bay were found to accumulate tributyltin. This accumulation did not appear to be an energy requiring process, since glucose did not significantly stimulate starved cells to accumulate tin.

INTRODUCTION

Tin has been an element of great importance to man's industries for millenia; organotin use by industrial societies more than doubled from 1966 to 1976 (ref.1). Tin's remarkable range of properties as organotin compounds has generated totally new markets and distribution patterns in the environment (ref.1), more than two-thirds being used as stabilizers for polyvinyl chloride plastics (ref.2); the remainder of the organotin compounds were incorporated into numerous biocidal materials as insecticides, herbicides, fungicides, and antifouling paints (refs.3,4).

As most tin compounds eventually are transported into the environment, i.e., soil and aqueous systems, it is extremely important, from an environmental and public health standpoint, to know the eventual fate of tin in these environments, especially its potential to become involved in food chains, as has happened with mercury as the result of the methylating activities of bacteria (ref.5). The question of the possible environmental methylation of tin is extremely important in view of the great increase in toxicity in going from the inorganic to the methylated forms; the order of animal toxicities of methyltin compounds (as chlorides, for example) is as follows: $(CH_3)_3SnCl > (CH_3)_4Sn > (CH_3)_2SnCl_2 > CH_3SnCl_3$, (ref.6).

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The first evidence for the methylation of tin by bacteria was obtained at the National Bureau of Standards in 1973 by Huey et al. (ref.7). A ubiquitous tin-resistant Chesapeake Bay bacterium (Ps. 244) growing in the presence of Sn(IV) was found to volatilize tin to a methyl species, as evidenced by the latter's capability to methylate Hg(II), and confirmed by mass spectral evidence. Several years later, the widespread occurrence of methylated tin species in natural waters (refs.8,9), rain, and human urine was demonstrated (ref.8). Recently, three groups have independently reported on the methylation of both inorganic tin and organotin substrates by the mixed population of microbial flora present in sediments collected from a Canadian fresh water lake (ref.10), estuarine sites in San Francisco Bay (ref.11), and the Chesapeake Bay (ref. 12).

The methodology for detection and speciation of environmentally occurring organotin compounds has advanced significantly since Huey et al. (ref.7) employed spectrofluorimetry, mass spectroscopy (MS), and observation of transmethylation of Hg(II) in the presence of Sn(IV) and Ps. 244 to propose the microbial methylation of tin. In the more recent reports the evolution and increasing sophistication of the methodology being utilized in the study of biogeochemical cycles of tin is evident. Braman and Tompkins (ref.8) described a technique for cryogenic trapping of organotins volatilized from aqueous solution by treatment with sodium borohydride. Detection was by flame emission using a hydrogen-rich, hydrogen-air flame. Hodge et al. (ref.9), while employing a similar hydride reduction and cryogenic trapping technique, used tin-specific atomic absorption detection in their work. Meinema et al. (ref.13) converted butyltin compounds to volatile butylmethyltin derivatives for separation and detection by a gas chromatograph-mass spectrometer system.

In the examples above, where aqueous samples were treated with sodium borohydride, no provision was made for isolation of Me_4Sn or other volatile organotins possibly present in the sample and not requiring hydride volatilization. The technique of Meinema et al. is not suitable for characterization of methyltin species. The objective of our work was to develop a technique capable of identification of methyltin species, including Me_4Sn, from aqueous environmental samples. We have applied a gas chromatographic system with a tin selective flame photometric detector to the speciation of the volatile methyltin compounds produced by Ps. 244 as originally reported, as well as speciation of organotin compounds in Chesapeake Bay waters. Additionally, the possible biotransformation of a prominent commercial organotin compound, tributyltin chloride, was investigated. The tributyltin moiety is one of the principal biocidal tin functions used in antifouling coatings.

TIN BIOMETHYLATION STUDIES

In our most recent studies of microbiological transformations of inorganic tin(II) and tin(IV) (ref.14), we have used the same Chesapeake Bay Ps. 244 as was employed in our earlier work. Respirant atmospheres above slants of Nelson's agar medium (ref.7), containing ten mg L^{-1} of tin(II) (as $SnCl_2$) or tin(IV) ($SnCl_4 \cdot 5H_2O$) (added after autoclaving) or free of tin (control), were analyzed by gas chromatography-mass spectrometry (GC-MS). Experiments were controlled with sterile and inoculated tubes of tin spiked and unspiked media, respectively.

The GC-MS system is interfaced with an on-line computer. This system provided two modes for data acquisition: mass spectrum mode and multiple ion detection (ion chromatogram) mode. The results of representative ion chromatograms of the respirant atmosphere above inoculated or sterile tin containing [ten ppm, Sn(IV)] slants after incubation for two weeks at ambient room temperature are shown in Fig. 1.

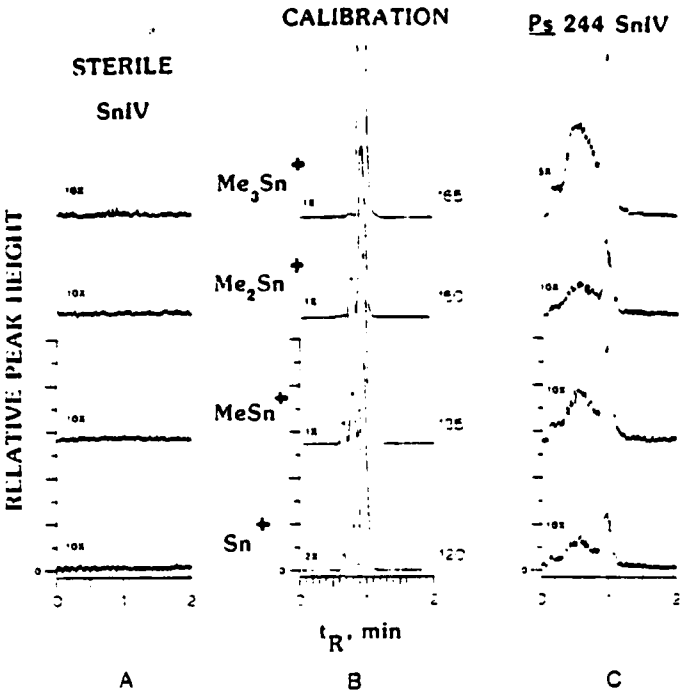

Fig. 1. Selected ion chromatograms obtained with GC-MS data system for (A) Headspace above sterile Nelson's agar slant media containing inorganic SnIV; (B) Calibration mixture of Me_2SnH_2, Me_3SnH, and Me_4Sn; (C) Respirant atmosphere above Ps. 244 inoculated Nelson's media containing with inorganic SnIV.

These ion chromatograms were obtained with the multiple ion detection mode of the GC-MS data system for all major fragment ions, including m/e 120, 135, 150, and 165 corresponding to the major tin isotopes in Sn^+, $MeSn^+$, Me_2Sn^{2+}; and Me_3Sn^+ multiplets, respectively. A calibration ion chromatogram of a mixture of Me_2SnH_2, Me_3SnH, and Me_4Sn, generated by the reduction of di- and trimethyltin chlorides with $NaBH_4$ in the presence of gaseous Me_4Sn, is also given to compare and identify the peaks from the respirant atmosphere above the Ps. 244 slants.

The ion chromatogram of the tin(IV)-containing Ps. 244 inoculated slant showed a methyltin compound at ~ 0.95 min, which corresponded to Me_4Sn in the calibration, as well as a very broad methyltin peak, obviously more volatile than Me_4Sn. The broadness of the peak indicates a decomposition reaction of methyltin species on the GC column as it was chromatographed. Comparison with the ion chromatogram shows an overlap of the broadened peak with the elution of Me_2SnH_2 and Me_3SnH in the calibration chromatogram. No tin species were detected in the atmosphere above

sterile controls containing with either tin(IV) (Fig. 1) or tin(II) (not shown). No peaks were detected from inoculated non-tin containing slants. These results basically confirm the previous work from this laboratory regarding the biomethylation of tin(IV) (ref.7) and that of the other investigators previously cited.

Repeated experiments using the Ps. 244 slants spiked with inorganic tin gave irreproducible production of methylstannanes. Because the solidified agar slants are inoculated on the surface, methylation of the tin in the medium would depend on the bioavailability of the tin, i.e., the proximity and molecular form of the tin at the surface of the agar slant. Recent studies (ref.25) indicate the non-uniform bacterial response to Sn(IV) in agar media.

Having determined the production of Me_4Sn and several methyltin hydrides (Me_nSnH_{4-n}, n = 2,3) by this organism, it was of interest to determine whether any of these compounds were naturally occurring in aquatic systems. Braman et al. (ref.8) and Hodge et al. (ref.9) reported the presence of Me_3Sn^+, Me_2Sn^{2+}, and $MeSn^{3+}$ species in aquatic systems. No Me_4Sn or methyltin hydrides were observed, however. We used a purge and trap gas chromatographic technique, with and without hydride reduction, to identify these volatile species (ref.14).

Water samples were collected in the early spring and late fall from the Chesapeake Bay and Baltimore Harbor in nitric acid washed glass bottles and stored at 2 to 4 °C until 10 to 15 minutes prior to analysis. For analysis, ten mL aliquots were transferred to 15 ml capacity, 16 x 125 mm borosilicate glass purge vessels and warmed to room temperature for analysis on the purge and trap-gas chromatograph-flame photometric detector system (P/T-GC-FPD). The 10 ml sample was purged with zero N_2 for 10 min (total purge volume = 200 mL), volatile trace elements being collected in the 60/80 mesh Tenax GC trap (Alltech. Assoc., Arlington Heights, IL) (ref.15).

Following the purge cycle, the trap was quickly heated to 250 °C for five minutes, to desorb the trapped volatiles, which were carried into the GC column. The flame photometric detector was modified to permit detection of SnH emission in a hydrogen-rich flame (ref.16) by using a 600 nm cut-on interference filter (bandpass 600 nm to 2000 nm) (Ditric Optics, Inc., Hudson, MA). The detector temperature was 200 °C. The signal from the FPD was recorded simultaneously on a strip chart recorder and on a digital integrator plotter. For the detection of non-volatile organotin species (cations), 100 to 500 µL of four percent $NaBH_4$ was added to the purge vessel immediately before initiating the ten min N_2 sample purge.

Results from analyses of samples collected during the spring month from a tributary receiving urban runoff in Baltimore Harbor (Jones Falls) gave evidence of the presence of tetramethyltin, as well as tin-containing species more volatile than Me_4Sn (Fig. 2). Borohydride was not added to the Bay sample for this analysis. Comparison with calibration chromatograms indicate that the amounts of methylstannanes in this sample were estimated to be (in ng L^{-1}): Me_2SnH_2 (200), Me_3SnH (400), and Me_4Sn (480). These were the highest estimates of methylstannanes

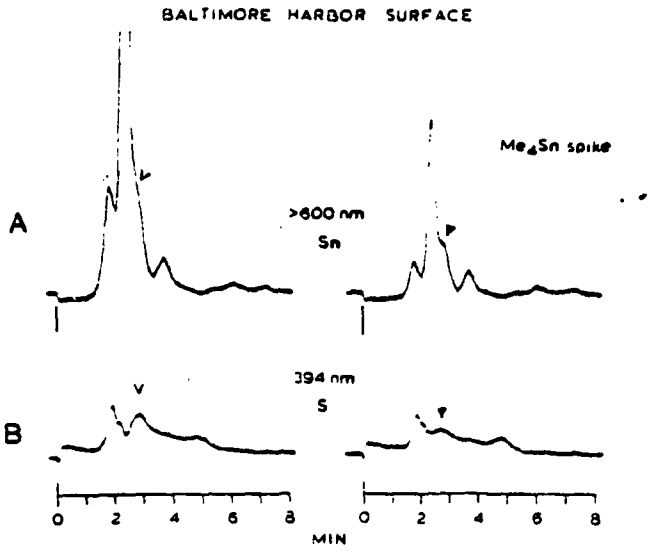

Fig. 2. Gas chromatograms obtained by P/T-GC-FPD method for 10 mL samples of Jones Falls surface water analyzed (A) in the tin-selective and (B) sulfur selective modes of the FPD, with and without Me_4Sn spike. Arrows denote Me_4Sn peaks.

speciated at this site in Baltimore Harbor. Tetramethyltin was also detected at another site in Baltimore Harbor (Colgate Creek) during the spring months. The samples collected at relatively pristine sites in the Chesapeake Bay at Tolly Point, Bloody Point, and Eastern Bay showed minimal amounts of methyltin species. These results appear to be the first observation of methyltinhydrides in the natural environment. Braman and Tompkins (ref.8) and Hodge et al. (ref.9) also reported the presence of methylstannanes, but these species were formed as a result of borohydride treatment of the sample after pre-hydride degassing, so that any of the volatile methylstannanes present would have been removed.

Production of methylstannanes by an aerobic microbe prevalent in the Chesapeake Bay along with the evidence of the presence of these compounds in the Bay does not demonstrate a direct causal relationship. What is shown is that the capacity for biogenesis of methyltins, in forms quite unexpected by organometallic chemists, may occur in environmental circumstances and that quantitative information concerning the stability and likely reactions of such reactive species in aqueous media is prerequisite to future studies on environmental tin chemistry. Although the biogenesis of Me_4Sn was seen to only occur with addition of Me_3Sn^+ to inoculated sediments (ref.10,11), the redistribution reactions of intermediate methyltins to form Me_4Sn by non-biological pathways must be noted as likely competitive events in such experiments (ref.11).

TRIBUTYLTIN DEGRADATION STUDIES

As tributyltin cation (TBT) is one of the most active biocidal agents extensively incorporated in antifouling coatings, its degradation in the environment to less toxic alkylated compounds appeared likely. The butyltins do not, however, readily lend themselves to analysis by hydridization and characterization by GC-FPD. At NBS, speciation of these compounds was accomplished through use of a high performance liquid chromatograph (HPLC) coupled to a graphite furnace atomic absorption

detector (GFAA) (ref.17). Through use of this system, the presence of dibutyltin in leachates of spent (removed by sand blasting) antifouling coatings, originally containing TBT, was found. Barug and Vonk (ref.23) also found dibutyltin to be formed in soils from TBT. It was of interest to determine what, if any, transformation of TBT could be brought about by bacteria.

Eight TBT-resistant bacterial isolates were obtained by plating sediment from three different sites in and near Baltimore Harbor on Nelson's medium (ref.7) containing 20 mg L^{-1} tin (as tributyltin chloride) and selecting morphologically distinct colonies for further purification. Three TBT-resistant strains, including Ps. 244, were obtained from Prof. R. R. Colwell (University of Maryland).

For the studies on the uptake of TBT, aliquots of log phase cultures of tributyltin-resistant bacteria (grown in 250 mL flasks containing 100 mL Nelson's medium at 28 °C and shaken at 200 rpm) were centrifuged, washed twice (sterile deionized water), and resuspended in 5.0 mM, pH 6.9 PIPES [piperazine-N-N-bis(2-ethanesulfonic acid)] buffer. After starvation in the buffer for 6 to 12 hours, the cells were resuspended in flasks containing fresh PIPES buffer plus tin (10 mg L^{-1} final concentration, as tributyltin chloride). Glucose was added to some of the suspensions, other batches of the cells were boiled. Aliqouts of the cell suspensions were removed with time and passed through 0.45 µm membrane filters (Millipore), which were digested in 0.2 mL HNO_3 in two mL plastic centrifuge tubes at 80 °C. The volume was made to 1.0 mL with deionized water and the digest was analyzed for tin by GFAA. In an early experiment with Ps. 244, cells were collected by centrifugation (12,000 x g, three min), washed twice in buffer, and digested as above.

For the studies on detection of possible tin metabolites, cultures of tributyltin-resistant bacteria were inoculated into 250 mL Mininert (Supelco) capped bottles or Erlenmeyer flasks containing 100 mL Nelson's broth and ten mg L^{-1} tin (as tributyltin chloride). Headspace gas from capped bottles was injected directly into the GC-FPD system described above. Aliquots of the culture medium from Erlenmeyer flasks were analyzed by $NaBH_4$ reduction-purge and trap techniques described above. In other experiments, certain isolates were grown in Nelson's broth plus tributyltin, and after eight days were centrifuged, washed twice in deionized water, then extracted with methanol. The methanol extract was analyzed for tributyltin using a HPLC-GFAA system (ref.17).

Initial studies indicated binding of TBT to the bacterial cells occurred (Fig. 3) mostly in the first hour of incubation. Starved cells and starved cells plus glucose showed similar uptake kinetics, indicating TBT accumulation was not a metabolically dependent function. Boiled cells accumulated about twice as much tin as live cells, suggesting that exclusion of TBT might be a mechanism of resistance, however, similar results have been previously reported with other metal-accumulating microorganisms (refs.18,19,20), and were attributed to additional cell envelope sites made available for binding metals. Yamada et al. (ref.21) suggested tripropyltin binds to phospholipids in the membrane of Escherichia coli. Several other tin-resistant Chesapeake Bay isolates were examined for tin accumulation. As with Ps. 244, all bound most tributyltin within the first 1.5 hours of incubation by a non-energy requiring process. If the cells on the filters were washed with a 5.0 mL aliquot of EDTA (1 mM) instead of PIPES buffer, 50 to 70 percent of the bound tin was removed (data not shown), indicating most of the tributyl-

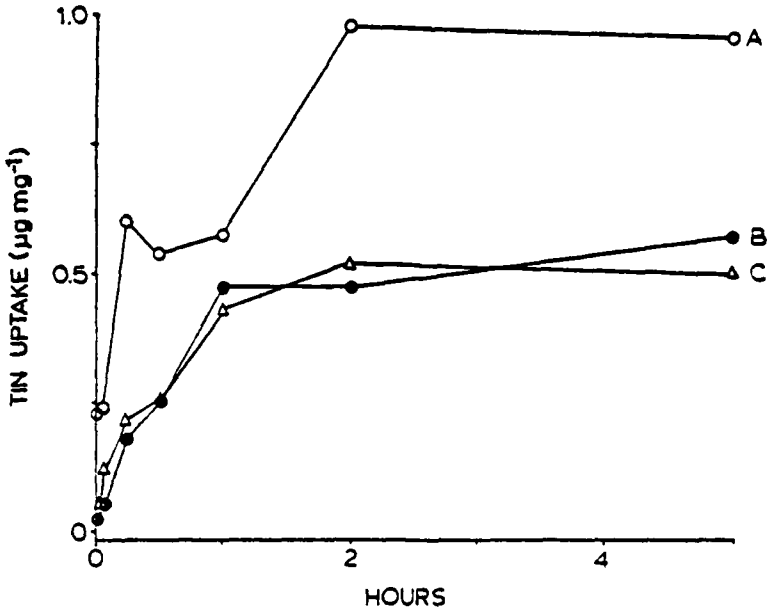

Fig. 3. Accumulation of tributyltin by Ps. 244. At time zero, tin (as Bu_3SnCl) was added (10 mg/L final conc.) to flasks containing 50 mL 5 mM PIPES buffer (pH 7.4) plus (A) Boiled cells (10 min), (B) Starved cells (10 hr, 22 °C in 5 mM PIPES buffer), (C) Starved cells plus glucose (10 mM final conc.)

tin was bound at the cell envelope and not inside the cells (ref.22). The isolates accumulated tin to 0.3 to 0.8 percent of their dry weight, resulting in dry weight concentration factors of 356 to 855.

Analysis of Nelson's medium containing tributyltin chloride and each of the tributyltin-resistant cultures by headspace and purge and trap gas chromatography revealed that no significant biotransformations of tributyltin occurred. In addition, HPLC-GFAA analysis of methanol extracts of some of the isolates, which had bound tributyltin, revealed the presence of only the tributyltin species. No degradation products, such as dibuyltin species, could be detected. Based on HPLC-GFAA system sensitivity, biotransformation of one percent of tributyltin in solution or three percent of cell bound tributyltin would have been detected.

Our results at NBS, therefore, show that a group of tributyltin-resistant estuarine bacteria accumulate but do not metabolize tributyltin in pure culture, which raises the question of biotransformation and persistence of tributyltin species in the estuarine environment, and bioaccumulation of the compound in food chains. It is difficult, however, to extrapolate laboratory results to the natural environment. Therefore, current investigations are focusing on the in situ biodegradation of tributyltin in waters and sediments from the Chesapeake Bay.

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11. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here)

The first evidence for the bacterial methylation of tin from inorganic Sn(IV) was presented in 1973 and recently has been verified by other investigators. Recent studies with the Pseudomonas strain, first employed in the initial observations, have indicated that the volatile methylated species produced by this organism from Sn(IV) and to a considerably lesser extent from Sn(II), include tetramethyl tin (Me_4Sn) and a number of hydritic methylstannanes (Me_nSnH_{4-n}, n = 2,3). Subsequently, using hydridization coupled with a new purge/trap GC-FPD method, methylated tin compounds (methylstannanes) have been found in the Chesapeake Bay, as both volatile and non-volatile species [$(CH_3)_nSnH_{4-n}$ and $(CH_3)_nSn^{(4-n)+}$, respectively]. Concentrations have ranged from 0 to 930 ng L^{-1}. Studies on the biological transformation of organotin compounds have so far indicated that there are no significant biotransformations of biocidal tributyltin (TBT) moiety. TBT-resistant organisms from the Chesapeake Bay were found to accumulate tributyltin. This accumulation did not appear to be an energy requiring process, since glucose did not significantly stimulate starved cells to accumulate tin. In studies with one of the organisms, killed cells were found to bind more than twice as much TBT as live cells, suggesting that exclusion of the TBT might be a mechanism of resistance.

12. KEY WORDS (Six to twelve entries; alphabetical order; capitalize only the first letter of the first key word unless a proper name; and separate key words by semicolons)

atomic absorption detector; bacterial accumulation; bacterial methylation; flame photometric detector; gas chromatography; high pressure liquid chromatography; methylstannanes; purge/and trap sampling; tetramethyltin; tin IV; Tin (II) tributltin

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